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Reaper is a central regulator of apoptosis in the fly, Drosophila melanogaster. At the start of this proposal our laboratory identified what was believed to be a pro-apoptotic human homolog of Reaper. This was of extreme interest as no true Reaper homolog had been identified in any organism. Accordingly, we proposed in the original statement of work to investigate the ability of the isolated gene to a) induce apoptosis upon overexpression and b) contribute to radiation-induced apoptosis. We then proposed to investigate expression of this protein in response to p53 in breast cancer cells and to monitor control of hrpr production and activity.

Task Ia. Investigate hrpr's ability to induce apoptosis when overexpressed:

We found that overexpression of hrpr could, indeed, induce apoptosis in human cells upon overexpression. However, this was not due to an intrinsic pro-apoptotic activity of the protein (see task 2b, below).

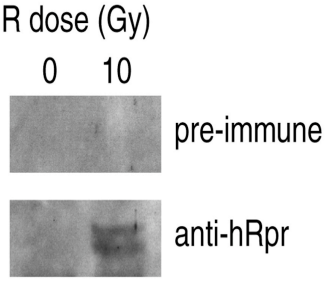

Fig. 1. ML-1 cells were irradiated with 10 Gray ionizing radiation and lysed in RIPA buffer. Lysates were immunoprecipitated and immunoblotted with anti-hrpr sera.

Task Ib. Investigate hrpr's contribution to radiation-induced apoptosis

We determined that hrpr was radiation-inducible at both the protein and mRNA level (see Fig 1 and 2). However, we did not continue investigation into its specific role in radiation-induced apoptosis, as explained in Task 2b, below.

Task 2a. Investigate the potential role p53 in hrpr induction

We found no change in hrpr expression following overexpression of p53 and saw no difference in radiation-inducibility in p53 positive or negative cells. Thus, this hypothesis was disproved.

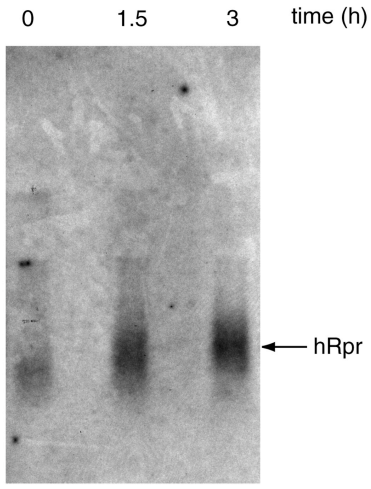

Fig. 2. Samples were treated as in Fig. 1 for the times indicated. mRNA was extracted and resolved on agarose gels for northern blotting with hRpr cDNA as a probe.

Task 2b. Investigate the role of translational control in hrpr production.

This turned out to be the most fruitful avenue of investigation. At the time we submitted this proposal, the elements of Reaper's primary sequence required for apoptotic induction was not well understood. However, during the course of this work, we undertook an in-depth study of Drosophila Reaper structure and function. As reported by many labs, it was found that the N-terminus of Reaper contained an IAP inhibitory domain and the central region of the protein contained a domain, known as the GH3 domain that confers mitochondrial localization and is required for full apoptosis. However, once these features of Reaper were well understood, it became apparent that the hrpr we had isolated contained neither an IAP inhibitory domain nor a GH3 domain. Rather, it shared homology with Reaper in a novel region which our laboratory later found

(see below) was critical for translational inhibition by Reaper. Hence, the pro-apoptotic activity we had observed in Task I, was actually the result of protein translation inhibition. Moreover, we found that the shared domain between hrpr and fly Reaper actually conferred binding to the 40S subunit of the ribosome, leading to marked inhibition of cap-dependent translation. Intriguingly, we have also found that hrpr is encoded by a bicistronic message (Fig. 3) and translated from an internal ribosome entry site, which makes sense given its ability to inhibit cap-dependent translation. Hence, we now believe that hrpr is a radiation-inducible translation inhibitor. In the course of this work, our lab also determined that the small non-structural proteins from Bunyaviruses responsible for host protein translational inhibition also shared homology with hrpr and Reaper. Hence, we have found an evolutionarily conserved motif that regulates protein translation through direct binding to the ribosome.

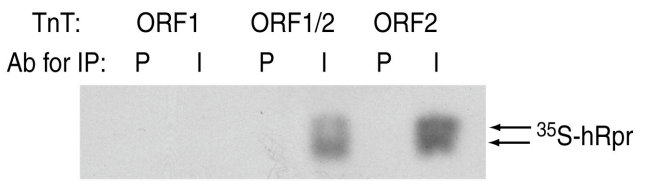

Fig. 3. The bicistronic message encoding hRpr (ORF2) and an unknown upstream ORF (ORF1) was translated in vitro with radiolabeled methionine and immunoprecipitated with anti-hRpr sera. Both ORF 2 alone and the bicistronic message (but not ORF1 alone) produced hRpr protein.

Task 2c. We believe that the two forms of hrpr observed at the time of this application result from alternative translational start sites rather than post-translational modification.

Key research accomplishments:

- Identification of critical domains of Reaper
- Demonstration of ability of Reaper to induce IAP autoubiquitination
- Demonstration of Reaper's ability to inhibit protein translation
- Characterization of hrpr as a novel human translational inhibitor

Reportable outcomes: (note that this project was undertaken by two different students as the first PI graduated and the award was reassigned. The published work below was authored by the first recipient of this award, Chris Holley. Work by the second recipient of this award, Jen Perry, is still underway. We also include an important publication (Colon-Ramos et al) that arose from this work.

Olson, M.R., Holley, C., Yoo, S.J. Huh, J.R. , Hay, B.A. and Kornbluth, S. (2003). Reaper is regulated by IAP- mediated ubiquitination. *J. Biol. Chem*, **278***: 4028-34.*

Olson, M.R., Holley, C., Gan, E.C., Colon-Ramos, D.A., Kaplan, B., and Kornbluth, S. (2003). A GH3- like domain in Reaper required for mitochondrial localization and induction of IAP degradation. *J Biol. Chem,* **278**: 44758-44768

Holley, C.,M. Olson, D. Colon-Ramos, and S. Kornbluth (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nature Cell Biol,* **4**: 439-444.

Colon-Ramos, D.A., C. Shenvy, D.H. Weitzel, E.C.Gan, R. Matts, J. Cate, and S. Kornbluth. (2006). Direct Ribosomal binding by a cellular inhibitor of translation. *Nature Structural and Molecular Biol*. **13**: 103-111.

Conclusion: We have learned a good deal about reaper function during the course of this work and have identified a novel regulator of translation in human cells that appears to be induced upon radiation and may contribute to death of breast cancer cells during radiation treatment through translational inhibition.